Insulin effects on sterol regulatory-element-binding protein-1c (SREBP-1c) transcriptional activity in rat hepatocytes

Dalila AZZOUT-MARNICHE, Dominique BÉCARD, Colette GUICHARD, Marc FORETZ, Pascal FERRÉ and Fabienne FOUFELLE[1]

U465 INSERM, Centre de Recherches Biomédicales des Cordeliers (Université Paris 6), 15, rue de l'Ecole de Médecine, 75270, Paris Cedex 06, France

The transcription factor sterol regulatory-element-binding protein-1c (SREBP-1c) plays a major role in the effect of insulin on the transcription of hepatic genes such as glucokinase and fatty acid synthase. We show here in cultured rat hepatocytes that insulin, through activation of the phosphatidylinositol 3-kinase pathway increases the abundance of the precursor form of SREBP-1c in endoplasmic reticulum. This precursor form is then rapidly cleaved, possibly irrespective of the continuous presence of insulin, leading to an increased content of the nuclear mature form of SREBP-1c. Nevertheless, the increased amount of the mature form of SREBP-1c in the nucleus is not a prerequisite for the rapid effect of insulin on the transcription of genes such as glucokinase, suggesting that additional actions of the hormone are involved, such as the activation of the nuclear form of SREBP-1c or of an unidentified SREBP-1c partner.

Key words: gene expression, glucokinase, PI3-kinase, transcription factor.

INTRODUCTION

We and others have shown that the transcription factor sterol regulatory-element-binding protein-1c (SREBP-1c) is involved in the stimulation by insulin of hepatic genes, which for their transcription are either exclusively dependent on insulin, such as glucokinase (GK), or dependent on insulin and glucose, such as fatty acid synthase, acetyl-CoA carboxylase, L-pyruvate kinase and Spot14 [1–3]. SREBP-1c belongs to a family of transcription factors involved in cholesterol and fatty acid metabolism [4]. It is synthesized as a precursor form (110 kDa) anchored in the endoplasmic reticulum and nuclear membranes. After proteolytic cleavage its mature active form (50 kDa) migrates into the nucleus where it can bind both sterol-regulatory elements (5′-TCACCCCCCAC-3′) and E-boxes (5′-CANNTG-3′) [5].

The mechanisms by which insulin stimulates the transcriptional activity of SREBP-1c are presently unknown. It is conceivable that insulin acts at different levels. We have already established that insulin stimulates SREBP-1c gene transcription in the liver [2] and this is also true in adipose tissue [1], although the insulin signalling pathway leading to increased transcription of the SREBP-1c gene is presently unknown. Insulin-stimulated SREBP-1c gene transcription should logically result in an increased abundance of the precursor form of SREBP-1c in membranes of the hepatic endoplasmic reticulum. By analogy with the regulation of SREBP-2 transcriptional activity, which involves a cholesterol-dependent proteolytic process [5], insulin could also enhance the proteolysis of the precursor form of SREBP-1c and thus its nuclear concentration. Indeed, SREBP-1c nuclear abundance is increased in the liver of starved mice after a refeeding period with a high carbohydrate diet or in the liver of streptozotocin diabetic rats injected with insulin [6,7]. Whatever the mechanism involved in the cleavage process, it is presently unknown whether an increased amount of the nuclear form of SREBP-1c is the sole mechanism by which insulin activates the transcription of its target genes. SREBP-1c is a very weak transcriptional activator when overexpressed in non-hepatic cell lines [8]. Some evidence suggests that the mature form of SREBP-1c could in fact be activated directly in the nucleus. When the mature form of SREBP-1c is overexpressed in 3T3-L1 adipocytes, its transcriptional activity is enhanced further by insulin [1]. It has been suggested that this mechanism involves a phosphorylation process by mitogen-activated protein kinase (MAP kinase) [9]. Insulin could then act on SREBP-1c in three different ways: increased gene transcription and protein synthesis, increased mobilization to the nucleus and activation of transcriptional activity.

Given the importance of the genomic actions of insulin on both carbohydrate and lipid metabolism, it is critical to understand the signalling pathways and mechanisms by which insulin regulates SREBP-1c activity and activates its target genes. In the present article, we have addressed the following questions. (i) Is the insulin-stimulated transcription of the SREBP-1c gene followed by increased amounts of both precursor and mature forms of SREBP-1c? (ii) Is the continuous presence of insulin necessary to activate the cleavage process? (iii) Is the increased amount of the mature form of SREBP-1c in the nucleus sufficient to explain the effect of insulin on its target genes, such as GK? (iv) What is the signalling pathway by which insulin activates the transcription of the SREBP-1c gene?

EXPERIMENTAL

Animals

Animal studies were conducted according to the French guidelines for the care and use of experimental animals. Female rats (200–300 g body weight) from Iffa-Credo (L'Arbresle, France) were used for isolation of hepatocytes. They were housed in plastic cages at a constant temperature (22 °C) with light from 07:00 h to 19:00 h for at least 1 week before the experiments. Hepatocyte isolation was performed at 09:00 h.

Hepatocyte isolation and culture

Hepatocytes were isolated from the liver of fed rats by the collagenase method [10]. Cell viability was assessed by the Trypan

Abbreviations used: GK, glucokinase; PI3-kinase, phosphatidylinositol 3-kinase; SREBP-1c, sterol regulatory-element-binding protein-1c; MAP kinase, mitogen-activated protein kinase; PKB, protein kinase B.

[1] To whom correspondence should be addressed (e-mail foufelle@bhdc.jussieu.fr).

Blue exclusion test and was always higher than 85 %. Hepatocytes were seeded at a density of 7×10^6 cells/dish in 100-mm Petri dishes in medium M199 with Earle's salts (Gibco BRL, Paisley, U.K.) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1 % (w/v) BSA, 2 % (v/v) Ultroser G (Gibco-BRL), 100 nM dexamethasone (Sigma, St. Louis, MO, U.S.A.), 1 nM insulin (Actrapid; Novo Nordisk, Copenhagen, Denmark), 100 nM tri-iodothyronine (T3; Sigma). After cell attachment (4 h), the medium was replaced by a medium similar to the plating medium but free of hormones (except when indicated), Ultroser and albumin. The cells were then cultured in various conditions as described in the Figure legends.

Isolation of total RNA and Northern-blot hybridization

Total cellular RNAs were extracted from cultured hepatocytes using the guanidine thiocyanate method [11] and prepared for Northern-blot hybridization as described previously [12]. Labelling of each DNA probe with [α-^{32}P]dCTP was performed by random priming (Rediprime labelling kit; Amersham Pharmacia Biotech, Little Chalfont, Bucks., U.K). SREBP-1c, GK and albumin cDNA were as described previously [3].

Preparation of membrane and nuclear extracts

Nuclear extracts were prepared from cultured hepatocytes by a modification of the procedure described by Dignam et al. [13]. Briefly, hepatocytes from 10 plates were broken with a tightly fitting Dounce A homogenizer in 10 mM Tris/HCl, 10 mM NaCl, 3 mM $MgCl_2$, 0.5 % Nonidet P40 and a mixture of anti-protease inhibitors (Complete™, Boehringer Mannheim, Mannheim, Germany). Nuclei were pelleted by a 10-min centrifugation (500 ***g***) at 4 °C and washed once in the same buffer. The nuclear pellet was resuspended in a hypertonic buffer (10 mM Hepes, pH 7.4, 0.42 M NaCl, 1.5 mM $MgCl_2$, 2.5 % glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol and the mixture of anti-protease inhibitors). Nuclei were allowed to swell for 30 min at 4 °C and a clear nuclear extract was obtained by centrifugation (100000 ***g***, 30 min, 4 °C). The supernatant from the initial low-speed centrifugation (500 ***g***) was further spun at 100000 ***g*** for 30 min to obtain a pellet of the crude membrane fraction. The pellet was resuspended in 10 mM Tris/HCl, pH 6.8, 100 mM NaCl, 1 % SDS, 1 mM EDTA and 1 mM EGTA.

Immunoblot analysis

Protein from nuclear extracts (30–60 μg) and from the membrane fraction (30–60 μg) from cultured hepatocytes were mixed with SDS loading buffer and subjected to SDS/PAGE on a 12 % gel. Protein content was determined as described by Bradford [14], using BSA as a standard. Proteins were electrotransferred on to Hybond C nitrocellulose membrane (Amersham Pharmacia Biotech). SREBP-1c was detected with a mouse monoclonal antibody (IgG-2A4) raised against amino acids 301–407 of human SREBP-1. IgGs were produced from a hybridoma cell line (A.T.C.C., Manassas, VA, U.S.A.) and purified from conditioned medium by Protein G–Sepharose affinity chromatography as described by the manufacturer (Amersham Pharmacia Biotech). Primary antibodies against SREBP-1c were used at 4 μg/ml and detection of signals was performed using the ECL Western-blot detection kit (Amersham Pharmacia Biotech) with anti-mouse horseradish peroxidase-conjugated IgG as second antibody.

Quantification of the results

Results are expressed as means ± S.E.M. and the significance was assessed using a paired Student's *t* test.

RESULTS AND DISCUSSION

We have shown previously that after 6 h of insulin stimulation, SREBP-1c mRNA content is strongly increased in cultured rat hepatocytes, whereas switching from a low (5 mM) to a high (25 mM) glucose concentration has no significant effect [2]. It is shown in Figure 1 that the insulin effect on SREBP-1c gene transcription is concomitant after 6 h with a 4.5-fold increase in the content of the SREBP-1c precursor form in cellular membranes and a 2.5-fold increase in the nuclear concentration of the SREBP-1c mature form. In contrast, a high glucose concentration has no significant effect. These results are in accordance with previous experiments *in vivo* showing that refeeding normal mice with a high-carbohydrate diet [6] or treating streptozotocin-diabetic rats with insulin [7] is concomitant with an increase in

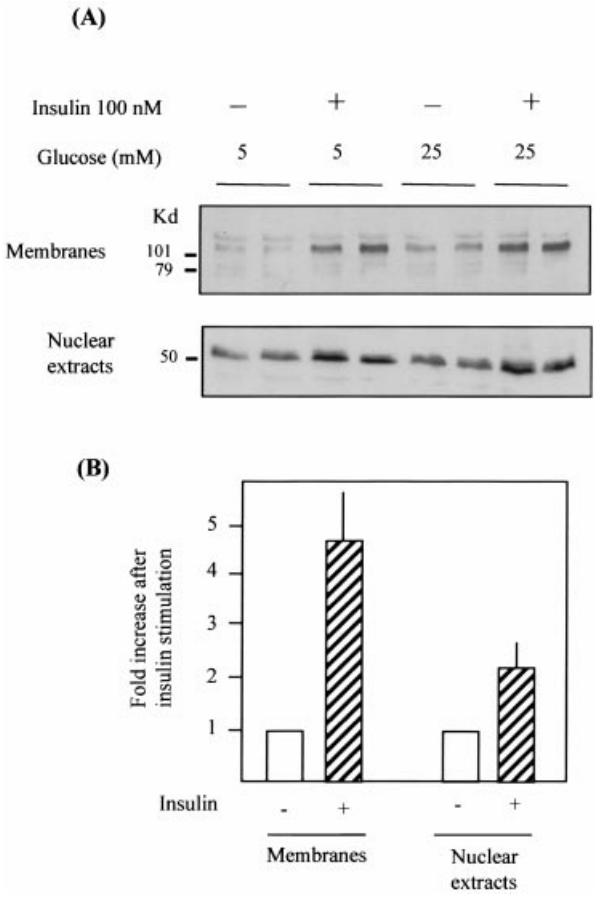

Figure 1 Changes of SREBP-1c precursor and mature forms in cultured hepatocytes in response to insulin and glucose

Hepatocytes were cultured 14 h after plating in the presence of 5 mM glucose. Cells were then cultured for 6 h in the presence of 5 or 25 mM glucose without or with 100 nM insulin. After 6 h, hepatocytes were scraped directly into lysis buffer (see the Experimental section) for preparations of nuclear and membrane extracts. Ten plates containing 7×10^6 hepatocytes were pooled for one condition. A representative Western blot (**A**) as well as the quantification of three independent experiments (**B**) are shown. The difference in SREBP-1c content in the presence or absence of insulin was significant at $P < 0.01$ and $P < 0.05$ respectively in membranes and nuclear extracts.

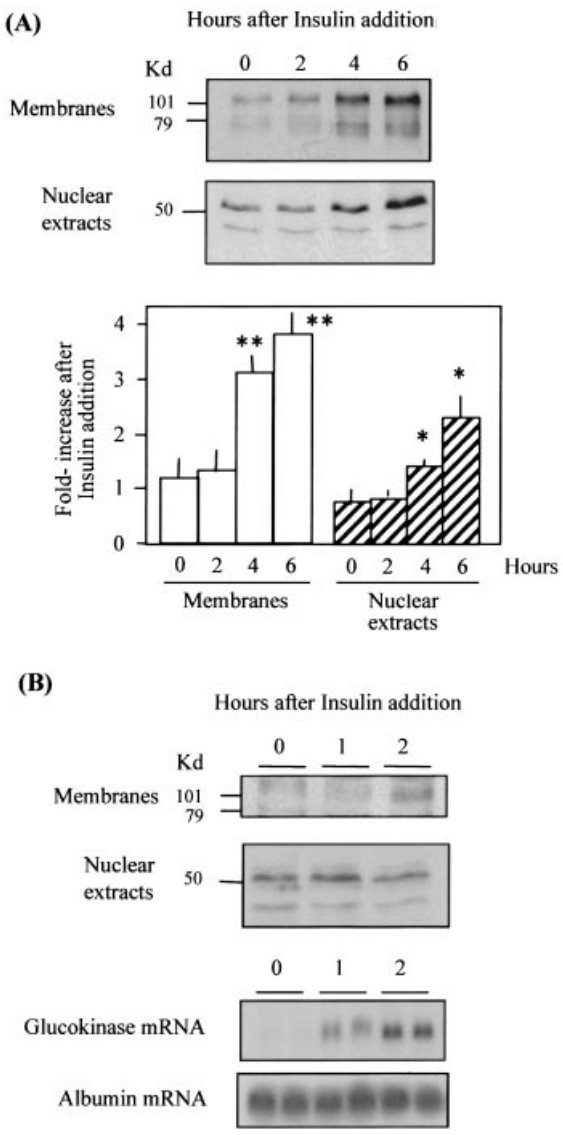

Figure 2 Time course of insulin action on the precursor and mature forms of SREBP-1c and GK expression in cultured hepatocytes

Hepatocytes were cultured 14 h after plating in the presence of 5 mM glucose. (**A**) Cells were then cultured for 2, 4 and 6 h in the presence of 5 mM glucose and 100 nM insulin. At each time, hepatocytes were scraped directly into lysis buffer (see the Experimental section) for preparations of nuclear and membrane extracts. Ten plates containing 7×10^6 hepatocytes were pooled for one condition. A representative Western blot is shown as well as the quantification of three independent experiments. * and ** show significant differences at $P < 0.05$ and $P < 0.01$ respectively when compared with time 0. (**B**) Cells were then cultured for 1 and 2 h in the presence of 5 mM glucose and 100 nM insulin. At each time, hepatocytes were scraped directly into lysis buffer (for preparations of nuclear and membrane extracts) or into guanidinium thiocyanate for extraction of total RNA. A representative Western blot as well as a representative Northen blot hybridized with GK and albumin probes from two independent experiments are shown.

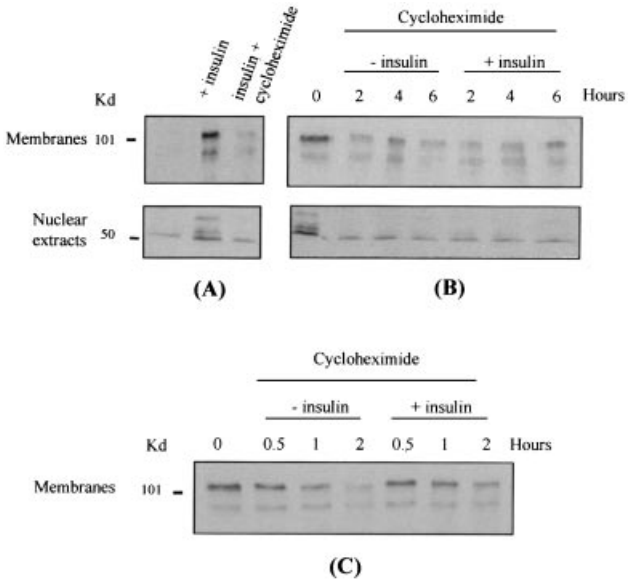

Figure 3 Time course of the disappearance of SREBP-1c precursor and mature forms after cycloheximide treatment in cultured hepatocytes

(**A**) In order to test the efficiency of the cycloheximide treatment, hepatocytes cultured overnight in the presence of 5 mM glucose and in the absence of insulin were then cultured for 6 h in the presence of insulin without or with 5 μM cycloheximide. After 6 h, hepatocytes were scraped directly into lysis buffer for preparations of nuclear and membrane extracts. A Western blot representative of two independent experiments is shown. (**B**) After plating, hepatocytes were cultured overnight in the presence of 5 mM glucose and 100 nM insulin in order to increase the amount of SREBP-1c precursor form in the membranes. Hepatocytes were then washed in PBS and incubated for 15 min in the presence of 5 μM cycloheximide. Hepatocytes were cultured for a further 2, 4 and 6 h without or with 100 nM insulin. (**C**) Experimental conditions were similar to (**B**) except that after cycloheximide addition, hepatocytes were cultured for 0.5, 1 and 2 h without or with 100 nM insulin.

the content of both precursor and nuclear mature forms of SREBP-1c.

Insulin action on gene expression is a rapid process. For instance, GK gene transcription, a SREBP-1c-dependent process [3], is activated by the hormone in less than 1 h in adult rat hepatocytes [15,16]. If one of the main actions of insulin on gene transcription is through an enhanced amount of the mature nuclear form of SREBP-1c, one would anticipate that this latter phenomenon is at least as rapid as the action of the hormone on gene transcription. We have thus performed experiments allowing us to detect in a time-dependent manner insulin-induced changes of the membrane and nuclear forms of SREBP-1c. After an overnight culture in the absence of insulin, the membrane content of SREBP-1c is low but still detectable (Figure 2A). The membrane content of SREBP-1c increases slightly 2 h after insulin addition. This increase is very significant after 4 h and maximal after 6 h (Figure 2A). An increase in the nuclear content of SREBP-1c is detectable only 4 h after insulin addition without early changes (Figure 2A) and is thus a rather delayed process.

In another set of experiments, we have compared the effect of insulin on membrane and nuclear content of SREBP-1c and the appearance of GK mRNA (Figure 2B). A slight increase in the membrane content of SREBP-1c is visible 2 h after insulin addition, whereas there is clearly no change in the nuclear content of mature SREBP-1c. In the same experiment, an increase in GK mRNA is visible at 1 and 2 h after insulin addition. The bulk of these results thus argue in favour of additional actions of insulin in order to explain its rapid effect on gene transcription. These effects could involve an activation of the residual amount of the mature form of SREBP-1c in the nucleus by a phosphorylation or dephosphorylation process. Alternatively insulin could act on an as-yet unidentified SREBP-1c partner.

We cannot infer from the previous experiments whether insulin has a direct effect on the proteolytic cleavage of the precursor

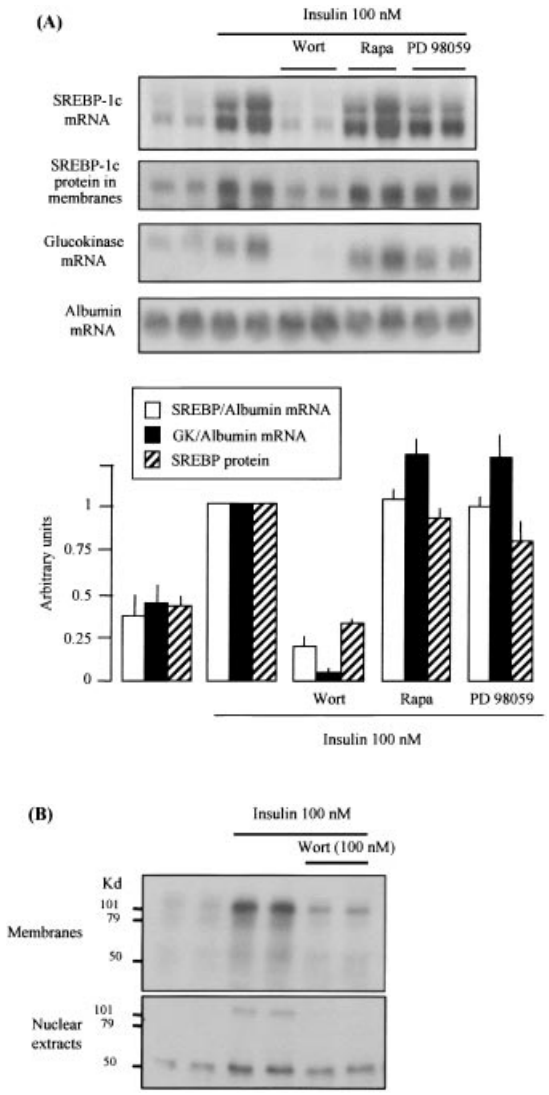

Figure 4 Effect of inhibitors of insulin signalling on SREBP-1c mRNA expression and SREBP-1c proteins in cultured hepatocytes

Hepatocytes were cultured 14 h after plating in the presence of 5 mM glucose. (**A**) Hepatocytes were then cultured for 6 h in the presence of 5 mM glucose and 100 nM insulin in the absence or presence of 100 nM wortmannin (Wort), 50 μM PD98059 or 50 nM rapamycin (Rapa) added 15 min before the addition of insulin. The carrier DMSO for the inhibitors was added in the control plates. At 6 h, hepatocytes were scraped directly into lysis buffer (see the Experimental section) for preparations of membrane extracts or in guanidinium thiocyanate for total RNA extraction. A representative Northern blot hybridized with SREBP-1, GK and albumin probes and a representative Western blot of three independent experiments are shown as well as their quantification in arbitrary units (the values in the presence of insulin were arbitrarily taken as 1 unit). The only significant differences when compared with the effect of insulin alone were observed in the presence of wortmannin ($P < 0.01$) (**B**) Hepatocytes were cultured for 6 h in the presence of 5 mM glucose and 100 nM insulin in the absence or the presence of 100 nM wortmannin added 15 min before the addition of insulin. The carrier DMSO was added to the control plates. At 6 h, hepatocytes were scraped directly into lysis buffer for preparations of nuclear and membrane extracts. A representative Western blot of two independent experiments is shown.

form of SREBP-1c. Indeed, the increased nuclear content of the mature form of SREBP-1c could also be the result of the increased membrane concentration of SREBP-1c precursor followed by a rapid constitutive proteolysis. In an attempt to solve this question, we have blocked the synthesis of SREBP-1c using cycloheximide and followed the kinetics of the disappearance of SREBP-1c in membranes and in the nucleus in the absence or presence of the hormone. At first, the efficiency of cycloheximide was tested by adding insulin to hepatocytes in the presence or absence of the drug. As seen in Figure 3(A), cycloheximide impairs the effect of insulin on the appearance of SREBP-1c in membranes and in the nucleus. In a second set of experiments, hepatocytes were cultured overnight in the presence of insulin in order to fill up the membranes with the precursor form of SREBP-1c. Cycloheximide was then added, and the hepatocytes were cultured for a further 6 h in the absence or presence of insulin (Figure 3B). After 2 h, the content of the precursor form of SREBP-1c in membranes and of the mature form in the nucleus is dramatically decreased irrespective of the presence of insulin and does not change for the next 4 h. Experiments showing shorter time points confirm that the decrease in the membrane content of SREBP-1c is not affected by the presence of insulin (Figure 3C).

These experiments suggest that insulin has no major effect on SREBP-1c proteolysis and points towards a constitutive proteolytic process. However, we cannot totally exclude that a proteolytic signal, once initiated by insulin, persists in the absence of the hormone for a time long enough to explain the rapid SREBP-1c disappearance in membranes. Nevertheless, this demonstrates that the proteolytic process is extremely rapid and that an ongoing SREBP-1c synthesis is necessary to maintain a high level of SREBP-1c in both the membrane and nuclear compartment. In addition, this shows that the nuclear half-life of SREBP-1c is short but that a residual amount of nuclear SREBP-1c still persists for several hours.

In vivo, the increase of SREBP-1c in membranes after a carbohydrate meal would last much longer owing to a more gradual decrease in insulin and SREBP-1c mRNA concentrations. The pool of SREBP-1c in the membranes would then act as a reservoir, allowing the presence of a significant amount of mature SREBP-1c in the nucleus long after the last meal. It is interesting to note that, in hepatocytes, and after a period of 14 h without insulin, detectable amounts of nuclear SREBP-1c are still present (see Figures 1 and 2).

We next addressed the question of the insulin-signalling pathway which might be involved in the effects of insulin on SREBP-1c-mediated gene transcription. Binding of insulin to its receptor induces autophosphorylation of the latter. Tyrosine-phosphorylated insulin receptor then phosphorylates insulin-receptor substrates IRS-1 and IRS-2 on tyrosine residues. This activates divergent signalling pathways, such as the MAP kinase and phosphatidylinositol 3-kinase (PI3-kinase) pathways. PI3-kinase activation, by producing phosphatidylinositol 3,4,5-trisphosphate, would lead in turn to the activation of protein kinase B (PKB)/Akt, either directly or indirectly through the activation of a 3-phosphoinositide-dependent serine/threonine kinase. PKB/Akt is one of the major downstream mediators of PI3-kinase. PI3-kinase can also activate p70 S6 kinase, which could account for the stimulation of protein synthesis by insulin. The PI3-kinase branch of the insulin signalling pathway seems to account for the metabolic action of insulin since it is involved in the stimulation of glycogen synthesis and glucose transport (GLUT4) by the hormone [17–19]. In order to analyse which insulin signalling pathway, MAP kinase or PI3-kinase, is involved in the stimulation of SREBP-1c gene expression, we have analysed the effects of insulin in the presence of various inhibitors.

The flavone compound PD98059 was without effect on insulin-induced SREBP-1c mRNA, SREBP-1c abundance in membranes and GK expression, indicating that the insulin effect is not mediated by the MAP kinase pathway (Figure 4A). In contrast,

the fungal metabolite wortmannin, a potent inhibitor of PI3-kinase, blocked the effects of insulin on SREBP-1c expression, and abolished the increased SREBP-1c abundance in membranes and in the nucleus as well as GK expression (Figure 4). This strongly suggests that the insulin effect is mediated through the PI3-kinase pathway. Similar results were obtained with a structurally distinct PI3-kinase inhibitor, the benzopyran-4-one LY294002 (results not shown). In this instance, p70 S6 kinase is not the downstream target of PI3-kinase since rapamycin does not affect insulin-induced SREBP-1c expression or its membrane abundance (Figure 4A). This suggests that the activation of PKB/Akt could be involved in the action of insulin. Interestingly enough, Wang and Sul [20] have shown in 3T3-L1 adipocytes that the effect of insulin on fatty acid synthase gene expression, which is also dependent on SREBP-1c, is mediated by the PI-3 kinase pathway, probably through the activation of PKB/Akt.

The results on GK gene expression (Figure 4A), which is dependent on SREBP-1c [3], also suggest that if insulin is activating the mature form of SREBP-1c or an unidentified partner, this effect is not mediated by the MAP kinase pathway since GK induction by insulin is not modified by PD98059. This is in contrast with results showing that MAP kinases were able to stimulate the transcriptional activity of SREBP-1c [9]. However, it must be pointed out that these results were obtained in cell lines by using ectopic overexpression of SREBP-1c and upstream activators of MAP kinase.

In conclusion, we have shown that insulin, through the PI3-kinase pathway (but not using the S6 kinase branch), activates the synthesis of the precursor form of SREBP-1c whereas a high glucose concentration has no significant effect. This precursor form is then rapidly cleaved, possibly by a constitutive process. The rapid effect of insulin on gene transcription does not require an increased amount of the mature form of SREBP-1c in the nucleus, suggesting that additional actions of insulin are necessary.

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